The functional interplay between the HIF pathway and the ubiquitin system – more than a one-way road

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Abstract: The hypoxia inducible factor (HIF) pathway and the ubiquitin system represent major cellular processes that are involved in the regulation of a plethora of cellular signaling pathways and tissue functions. The ubiquitin system controls the ubiquitination of proteins, which is the covalent linkage of one or several ubiquitin molecules to specific targets. This ubiquitination is catalyzed by approximately 1000 different E3 ubiquitin ligases and can lead to different effects, depending on the type of internal ubiquitin chain linkage. The best-studied function is the targeting of proteins for proteasomal degradation. The activity of E3 ligases is antagonized by proteins called deubiquitinases (or deubiquitinating enzymes), which negatively regulate ubiquitin chains. This is performed in most cases by the catalytic removal of these chains from the targeted protein. The HIF pathway is regulated in an oxygen-dependent manner by oxygen-sensing hydroxylases. Covalent modification of HIF subunits leads to the recruitment of an E3 ligase complex via the von Hippel-Lindau (VHL) protein and the subsequent polyubiquitination and proteasomal degradation of HIF subunits, demonstrating the regulation of the HIF pathway by the ubiquitin system. This unidirectional effect of an E3 ligase on the HIF pathway is the beststudied example for the interplay between these two important cellular processes. However, additional regulatory mechanisms of the HIF pathway through the ubiquitin system are emerging and, more recently, also the reciprocal regulation of the ubiquitin system through components of the HIF pathway. Understanding these mechanisms and their relevance for the activity of each other is of major importance for the comprehensive elucidation of the oxygen-dependent regulation of cellular processes. This review describes the current knowledge of the functional bidirectional interplay between the HIF pathway and the ubiquitin system on the protein level.

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The functional interplay between the HIF pathway and the ubiquitin system – more than a one-way road

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Abstract

The hypoxia inducible factor (HIF) pathway and the ubiquitin system represent major cellular processes that are involved in the regulation of a plethora of cellular signaling pathways and tissue functions. The ubiquitin system controls the ubiquitination of proteins, which is the covalent linkage of one or several ubiquitin molecules to specific targets. This ubiquitination is catalysed by approximately 1000 different E3 ubiquitin ligases and can lead to different effects, depending on the type of internal ubiquitin chain linkage. The best-studied function is the targeting of proteins for proteasomal degradation. The activity of E3 ligases is antagonised by proteins called deubiquitinating enzymes (or deubiquitinases), which negatively regulate ubiquitin chains. This is performed in most cases by the catalytic removal of these chains from the targeted protein. The HIF pathway is regulated in an oxygen-dependent manner by oxygen-sensing hydroxylases. Covalent modification of HIFα subunits leads to the recruitment of an E3 ligase complex via the von Hippel-Lindau (VHL) protein and the subsequent polyubiquitination and proteasomal degradation of HIFα subunits, demonstrating the regulation of the HIF pathway by the ubiquitin system. This unidirectional effect of an E3 ligase on the HIF pathway is the best-studied example for the interplay between these two important cellular processes. However, additional regulatory mechanisms of the HIF pathway through the ubiquitin system are emerging and, more recently, also the reciprocal regulation of the ubiquitin system through components of the HIF pathway. Understanding these mechanisms and their relevance for the activity of each other is of major importance for the comprehensive elucidation of the oxygen-dependent regulation of cellular processes. This review describes the current knowledge of the functional bidirectional interplay between the HIF pathway and the ubiquitin system on the protein level.

Keywords: Hypoxia, PHD, FIH, deubiquitinase, E3 ligase, inflammation

1 Introduction

Tissues and cells require a sufficient supply of oxygen for their metabolic needs to produce the appropriate amount of energy for all necessary cellular biological processes to occur [1-3]. If the cellular oxygen demand is not met by its supply (hypoxia), the cells have to adjust in order to survive [1, 3]. Hypoxia occurs throughout a wide range of physiological and pathophysiological conditions, such as development, cardiovascular disease, chronic inflammation and cancer [2, 4, 5]. Therefore, it is vital for cells to be able to continuously "sense" their local, available oxygen levels. The hypoxia-inducible factor (HIF) pathway is the major signaling pathway responsible for cellular oxygen sensing and adaptation to hypoxia [1, 2].

The ubiquitin system provides a key cellular mechanism for the regulation of protein fate and function [6, 7]. Most signaling pathways and cellular processes are affected by the ubiquitin system and thousands of proteins are ubiquitinated within cells [8]. The ubiquitin system impacts on biological processes through the regulation of protein degradation, interaction, localization and activity [8].

This review describes the functional interplay between these two major cellular processes. We focused on the mutual, direct regulation between the two main constituents of the ubiquitin system, the E3 ubiquitin ligases and the deubiquitinases, and the components of the HIF pathway on the protein level.

1.1 The HIF pathway

Four different cellular oxygen sensors are currently known. All of them are protein hydroxylases that belong to the Fe(II)- and 2-oxoglutarate-dependent dioxygenase superfamily and use molecular oxygen as an essential co-substrate [1, 9]. Beside Fe(II), a reducing agent such as ascorbate is required as co-factor by the hydroxylases [10]. However, ascorbate itself is dispensable and can be replaced for example by glutathione [11]. Three of the hydroxylases are prolyl-4-hydroxylase domain (PHD) proteins 1-3 and one is the asparagine hydroxylase factor inhibiting HIF (FIH) [1]. These enzymes regulate the

heterodimeric HIF transcription factor through hydroxylation of its three α subunits (HIF-1α, -2α, -3α) [1, 12]. In normoxia, prolyl-4-hydroxylated HIFα is bound by the von Hippel-Lindau (VHL) protein, the ligand-recognizing component of the E3 ubiquitin ligase cullin 2/elongin B & C/Rbx-1 (RING-box protein 1) complex [13-17]. Following its recruitment through VHL, the E3 ligase catalyzes the polyubiquitination of HIFα, leading to its proteasomal degradation [13-18]. FIH-dependent asparagine hydroxylation of HIFα inhibits its interaction with the transcriptional co-activators p300/CBP, attenuating HIF transactivation activity [1, 2]. In hypoxia, the molecular oxygen availability is limited for the hydroxylases, reducing the number of HIFα hydroxylation events [1]. Stabilized HIFα forms together with HIF-1β/ARNT the active heterodimeric transcription factor HIF. Both subunits recruit transcriptional co-activators, including histone acetyltransferases, and enhance the expression of specific genes, leading to the adaptation of cells to hypoxia [2]. Lorenz Poellinger majorly contributed to the elucidation of the function of ARNT as important component of the HIF heterodimer and the recruitment of p300/CBP as co-activators [19-21]. Furthermore, his group was among the pioneers demonstrating that HIF-1α is regulated through polyubiquitination and the ubiquitin-proteasome pathway [18]. These findings opened up the field of the HIF pathway for the analysis of its functional interplay with the ubiquitin system.

1.2 The ubiquitin system

The post-translational, covalent attachment of ubiquitin proteins (Ub's) to substrate proteins is called ubiquitination [8]. Ub's are attached to their targets through a concerted mechanism involving ubiquitin-activating enzymes (E1s), ubiquitin-conjugating enzymes (E2s) and ubiquitin ligases (E3s) (Fig. 1) [22]. An isopeptide bond is formed between an Ub and a substrate protein, leading to monoubiquitination [8]. Subsequently, further ubiquitin proteins can be linked to the attached Ub, leading to polyubiquitination. Mono- and polyubiquitination can have diverse effects, depending on the linkage sites between the Ub proteins (Fig. 1). Seven different lysyl residues (K 6, 11, 27, 29, 33, 48, 63) as well as the N-terminus (M 1) of an Ub protein can be used for the formation of Ub chains [8]. Ub chains can contain only one

specific or several different types of linkages, which differently affects their three-dimensional structure [8, 22]. Furthermore, one Ub protein can be modified with several other Ub proteins, leading to branched Ub chains [8]. Subsequent binding of proteins containing ubiquitin-binding domains (UBDs) to specific Ub chains translates the diverse chain structures into different downstream effects [8]. For example, Ub chains linked through K48 can target substrate proteins for proteasomal degradation. A prime example for a K48 Ub chain-dependent regulation of protein degradation is the prolyl-4-hydroxylation-dependent, VHL-mediated polyubiquitination and subsequent proteasomal degradation of HIF-1α [1]. K63-linked and linear (M1-linked) Ub chains in turn play, among other processes, an important role in signal transduction, serving as recruitment scaffolds for downstream signaling proteins, e.g. in inflammatory pathways [22].

The outcome of Ub chain modifications is not only dependent on the type of attached Ub chain. Another key aspect for downstream events is the selection of specific substrate proteins. This is regulated through the E3 ligases [23]. Of note, Ub chains that are not attached to substrates have also been identified (referred to as unanchored Ub chains). These chains serve as recruitment platforms for signaling proteins [8]. It is estimated that over 1000 E3s exist within a cell, alongside 35 E2s and 2 E1 enzymes [6, 7]. E2s are responsible for adequate Ub conjugation and can influence which Ub lysine residue is used during Ub chain formation, affecting the outcome of downstream signaling events [7]. E1 enzymes are responsible for the ATP-dependent activation of Ub for the subsequent ubiquitination event through E2s and E3s [6].

Like many other post-translational modifications, ubiquitination is reversible [23]. A superfamily of isopeptidases has been identified as negative regulators of ubiquitination, called deubiquitinases (DUBs; also called deubiquitinating enzymes), counteracting the function of E3 ligases [23-25]. Approximately 100 DUBs are encoded in the human genome. These can be divided into seven subfamilies based on their structures: ubiquitin-specific proteases (USPs), ubiquitin C-terminal hydrolases (UCHs), ovarian tumor proteases (OTUs), Josephins, the JAB1/MPN/MOV34 family (JAMMs), monocyte chemotactic protein-induced

proteins (MCPIPs) and the motif interacting with Ub-containing novel DUB family (MINDYs) [23, 24, 26]. All DUBs of these subfamilies are cysteine proteases except the JAMM subfamily members, which are zinc metalloproteases [23, 26]. Removal of Ub chains from proteins by DUBs prevents proteasomal degradation, leading to the stabilization of these proteins or negatively regulates signaling pathways through removal of the recruitment sites (Ub chains) for downstream proteins (such as in NF-κB signaling pathways) [22, 23]. DUB activity can further contribute to Ub homeostasis through recycling of ubiquitin from proteins that are degraded [23]. Overall, DUBs affect many different signaling pathways and key biological processes, which has sparked major interest in their function, the regulation of their activity as well as in their potential as novel therapeutic targets.

2 Functional interplay between the HIF pathway and E3 ligases

2.1 Hydroxylases and E3 ligases

Beside the direct regulation of HIF hydroxylase activity through the availability of O_2, their abundance is also of major importance for their impact on the HIF transcription factors. This was highlighted by the observation that the expression of PHD2 and PHD3 is regulated by HIF, leading to increased PHD2 and PHD3 protein levels in hypoxia, reducing HIFα protein levels and serving as a negative feedback loop in hypoxic conditions [27]. E3 ligases catalyzing the attachment of K48-linked Ub chains can affect the half-life and protein amount of the HIF hydroxylases and hence indirectly impact on the extent of the HIF transcription factor level and activity (Fig. 2A). But these hydroxylases have also been demonstrated to affect signaling pathways other than the HIF pathway, which might also be affected by the regulation of hydroxylase abundance [28-31].

The seven-in-absentia homologue (Siah) RING-type E3 ubiquitin ligases Siah1 and Siah2 regulate the stability of the HIF hydroxylases PHD1, PHD3 and FIH through polyubiquitination [32-37]. Of note, the human genome encodes for two Siah homologues (Siah1 and Siah2), whereas in mice three functional Siah genes have been identified (Siah1a, Siah1b, Siah2) [38, 39]. The substrate-binding domain (SBD) of human Siah2

shows a high affinity for PHD3, while the SBD of human Siah1 only weakly interacts with it [35]. But in human glioma cells also Siah1 has been linked to the regulation of PHD3 protein levels and in the human breast cancer cell line MCF7 both Siah1 and 2 are involved in PHD3 protein degradation [36, 37]. Silencing of either one of the two Siah E3 ligases resulted in increased PHD3 protein levels [36]. Murine Siah1a and Siah2 have both been reported to regulate the protein levels of PHD1 and PHD3, but the destabilizing effect of Siah2 for PHD1 and PHD3 was stronger [32]. Furthermore, Siah2-dependent regulation was higher for PHD3 than for PHD1 and PHD3 exhibited also a stronger affinity for Siah2 [32]. The higher susceptibility of PHD3 for Siah2-mediated degradation has in part been attributed to a missing N-terminal region in PHD3, which is present in PHD1 and PHD2 [33]. Interestingly, vitamin K3 inhibits Siah2, leading to increased PHD3 and decreased HIF-1α protein levels [40]. Of note, Siah1 E3 ligase activity regulates besides the PHDs also FIH protein levels [34]. In hypoxia, genetic deletion of murine Siah2 led to an increased half-life of PHD3 and decreased HIF-1α levels, while combinatorial deletion of Siah1a and Siah2 completely abrogated hypoxia-dependent HIF-1α stabilization [32]. Furthermore, Siah2-mediated degradation of PHD3 was increased in hypoxia through increased Siah2 transcript levels [32]. However, in the human breast cancer cell line MCF7, hypoxia did not affect Siah1 or Siah2 protein levels [36]. Moreover, Siah2-mediated degradation of PHD3 was increased in hypoxia through p38 MAPK-dependent Siah2 phosphorylation, indicating a different mechanism for a hypoxia-dependent regulation of Siah2 [41]. Overall, the regulation of HIF hydroxylases through Siah E3 ligases seems to be complex and context-specific and it would be of interest to identify the mechanisms leading to the contrasting observations.

PHD1 is also regulated by the cullin 3-based E3 ligase speckle-type POZ protein (SPOP). SPOP mediates PHD1 polyubiquitination and proteasomal degradation, and increased PHD1 levels and loss of SPOP were linked to prostate cancer growth [42]. Studies of zebrafish proteins demonstrated that the E3 ligases mindbomb (mib) mib and mib2 interact with and polyubiquitinate FIH, but the relevance and outcome of this modification is unclear [43, 44].

While most of the hydroxylases have been reported to be regulated by E3 ligases, it is noteworthy that for PHD2, the main regulator of HIFα, no E3-dependent regulation has to our knowledge been reported so far. Furthermore, this is in agreement with our previous work demonstrating that the ubiquitin system is dispensable for the regulation of PHD2 protein levels and that PHD2 is regulated through the prolyl cis/trans isomerase FKBP38 and the proteasome [45, 46]. It is intriguing that all hydroxylases seem to be regulated by the ubiquitin system but the key regulator of HIFα is not. It remains to be determined why evolution led to this apparent deviation of the regulation of the HIF pathway by the ubiquitin system at this critical signaling node.

Besides the regulation of the hydroxylases through E3 ligases, a reciprocal regulation has also been proposed. The ankyrin repeat and SOCS box protein 4 (ASB4), the substrate-recognizing protein of the cullin/elongin B & C/Roc ubiquitin ligase complex, has been reported to be hydroxylated by FIH and it was postulated that ASB4 hydroxylation promotes its substrate binding [47].

2.2 HIF transcription factor subunits and E3 ligases

The best-characterized E3-dependent regulatory mechanism of HIFα subunits is its VHL-dependent regulation and has been expertly reviewed elsewhere [15-17]. Beside this mechanism, other E3 ligases have also been reported to regulate HIFα . Their interaction with the HIF pathway on the protein level is the focus of this chapter (Fig. 2B). Of note, a recent investigation of VHL-independent degradation of HIF-1α in hypoxia indicates that ubiquitination is absolutely required for this regulation, highlighting further the importance of the interplay of the HIF pathway and the ubiquitin system [48]. To our knowledge, the regulation of E3 ligases by HIF through protein:protein interaction has not been reported so far.

Several E3 ligases affect HIF subunits through mechanisms involving heat shock proteins. We and others have previously shown that heat shock protein 90 (Hsp90) binds to HIF-1α, affecting its stabilization [19, 49-53]. The receptor of activated protein C kinase

(RACK1) mediates HIF-1α degradation in an oxygen-independent manner [54-57] and competes with Hsp90 for its HIF-1α binding site, recruiting the cullin 2/elongin B & C/Rbx-1 E3 ligase complex to target HIF-1α for proteasomal degradation [54, 55]. As negative regulator of this mechanism the mammalian septin family member SEPT9_v1 has been described, a specific interactor of HIF-1α but not HIF-2α, that prevents the HIF-1α interaction with RACK1 [56]. A second negative regulator is the proteolytically released intracellular domain of the receptor-tyrosine kinase ErbB4 that interacts with HIF-1α and suppresses RACK1-dependent HIF-1α degradation [58]. The E3 ligase cullin 5 has also been implicated in an Hsp90-mediated, oxygen-independent regulation of HIF-1α protein levels [59]. The E3 ligase CHIP (carboxyl terminus of Hsc70-interacting protein), also known as STUB1 (STIP1 homology and U-box containing protein 1), regulates the proteasomal degradation of HIF-1α in an Hsp70-dependent manner [60]. This regulation is independent of HIF-1α hydroxylation and is mediated by the interaction of Hsp70 with HIF-1α and the subsequent recruitment of CHIP [60]. Of note, this Hsp-mediated mechanism stands in contrast to the RACK1-dependent regulation of HIF, which depends on the competition for binding sites with Hsp90 and not its recruitment. This demonstrates different modes of actions for the functional interplay of Hsp's and E3's in the regulation of HIF. Interestingly, Hsp70- and CHIP-mediated degradation was selective for HIF-1α, mediating its degradation during prolonged hypoxia [60]. CHIP is also involved in the regulation of HIF-1α degradation during high glucose conditions (such as diabetes) when methylglyoxal (MGO) accumulates, leading to rapid degradation of HIF-1α in hypoxia [61]. MGO stimulates the association of HIF-1α with HSP40/70, enhancing the recruitment of CHIP and resulting in HIF-1α polyubiquitination followed by proteasomal degradation [61]. During nutrient deprivation, CHIP induces the conjugation of K63-linked polyubiquitin chains to HIF-1α, targeting it for chaperon-mediated autophagy [62, 63]. The CHIP-dependent regulation of HIF was further investigated as potential therapeutic target. Overexpression of microRNA miR-21 in human umbilical cord blood-derived mesenchymal stem cells (UCBMSCs) led to decreased CHIP levels,

increasing HIF-1α and neovascularization in critical limb ischemia [64]. In conclusion, Hsp's play an important role in the E3-dependent regulation of HIF, indicating a more general mechanism.

Beside the Hsp-mediated recruitment of E3 ligases to HIFα, also phosphorylation mediates E3 binding. The E3 ligase F-box/WD repeat-containing protein 7 (Fbw7) is recruited to HIF-1α through HIF-1α phosphorylation by glycogen synthase kinase-3 (GSK-3), leading to HIF-1α proteasomal degradation [65, 66].

For other E3 ligases, it is unknown if the recruitment to HIFα is regulated. The kelch-like 20 protein (KLHL20; also called KLEIP) is part of an E3 ligase complex and is induced by HIF-1 [67]. In addition, it directly interacts with HIF-2α but not HIF-1α, affecting HIF-2α protein levels [68]. The molecular mechanism of the KLHL20-dependent regulation of HIF-2α is unclear. Of note, KLHL20 knockout mice die due to respiratory failure, which was associated with reduced HIF-2α and VEGF mRNA levels in the embryonic lung [69]. The E3 ligase hypoxia-associated factor (HAF), also known as squamous cell carcinoma antigen recognized by T cells (SART1), targets HIF-1α but not HIF-2α for degradation independent of the oxygen level [70]. Interestingly, HAF is also capable of binding HIF-2α, but this association results in increased HIF-2 transcriptional activity [71, 72]. Therefore, it has been suggested that HAF mediates a switch from HIF-1 to HIF-2 target genes, eventually promoting tumor progression [71-74]. In the clear cell renal cell carcinoma (ccRCC) cell lines RCC4 and RCC10, the mutant VHL prevents HAF-dependent degradation of HIF-1α, presumably through competition for HIF-1α binding sites [75].

Interestingly, mouse double minute 2 homolog (Mdm2; also called Hdm2), a major regulator of the tumor suppressor p53, and breast cancer susceptibility gene 1 (BRCA1), a tumor suppressor itself, have both been associated with the regulation of HIF subunits. Mdm2 decreases HIF-1α protein levels through HIF-1α polyubiquitination and proteasomal degradation, but in contrast to this Mdm2-dependent regulation was also connected with increased HIF-1α protein levels and activity [76-82]. Mdm2 has been described to directly

bind to HIF-1α but others found that Mdm2 is recruited to HIF-1α through p53 [76, 80, 81, 83-85]. Another member of the p53 gene family, the transcription factor TAp73, also recruits Mdm2 to HIF-1α, leading to its proteasomal destruction independent of cellular oxygen levels [82, 86]. The functional interplay between Mdm2 and HIF-1α has been linked to the regulation of tumor angiogenesis [76, 82, 86]. The regulation of HIF by Mdm2 is interesting as it connects the regulation of both the HIF and the p53 pathway. But the involved mechanism seems to be complex and might be context specific. The E3 ligase BRCA1 is frequently mutated in breast cancer and has been described to regulate HIF-1α protein levels in breast cancer cells [87]. BRCA1 directly interacts with and stabilizes HIF-1α through inhibition of HIF-1α proteasomal degradation [87]. But it is unclear if BRCA1-dependent ubiquitination is involved in this mechanism [87]. However, in stromal fibroblasts BRCA1 knockdown led to upregulation of HIF-1α, indicating a cell type specific regulation [88]. In breast cancer tumors BRCA1 mutations were associated with increased HIF-1α levels [89-91].

HIF-1α polyubiquitination has mainly been described as the conjugation of K48-linked Ub chains required for the subsequent targeting for proteasomal degradation. But the E3 ligase TNF receptor-associated factor 6 (TRAF6) mediates the conjugation of HIF-1α with K63-linked polyubiquitin chains [92, 93]. While the above described CHIP-induced K63-linked polyubiquitination of HIF-1α results in its chaperon-mediated autophagy, TRAF6-dependent K63 polyubiquitination stabilizes HIF-1α by preventing its proteasomal degradation [63, 92, 93]. Furthermore, the E3 ligase Pellino3 has been described as negative regulator of TRAF6-mediated HIF-1α stabilization and HIF-dependent transcription [93]. No indications have been found for a TRAF6-dependent regulation of HIF-2α [92]. Moreover, TRAF6 expression and its activity have been reported to be increased by hypoxia, mediating the monoubiquitination of histone H2AX [94]. This in turn leads to the recruitment of ataxia-telangiectasia mutated (ATM) and the subsequent phosphorylation of H2AX (γH2AX) [94]. HIF-1α specifically interacts with γH2AX preventing HIF-1α nuclear export and degradation,

enhancing HIF-mediated gene expression [94]. Because TRAF6 is an important signaling component of major pro-inflammatory signaling pathways (such as the IL-1β signaling pathway), the observed interaction between TRAF6 and HIF-1α indicates that TRAF6 is a novel important signaling node for the integration of inflammatory signaling and the HIF pathway. It will be of interest to investigate whether the observed TRAF6:HIF-1α interaction affects inflammatory signaling.

3. Functional interplay between the HIF pathway and deubiquitinases

3.1 Hydroxylases and deubiquitinases

Recent investigations have shed first light on the functional interaction and potential mutual regulation of oxygen-sensing hydroxylases and deubiquitinases (Fig. 2A). Investigating the effect of hypoxia on NF-κB activity, it has been observed that the stability of the deubiquitinase CYLD is regulated by the human papillomavirus (HPV)-encoded protein E6 in an oxygen-dependent manner [95]. Hypoxia stimulated an E6-dependent ubiquitination and subsequent degradation of CYLD in HPV-infected cells, activating in turn NF-κB [95]. While the underlying mechanisms remained unclear, protein hydroxylation has been postulated to play a role [95].

We previously identified several proteins of the IL-1β signaling pathways as potential targets of PHD1 and FIH, including the deubiquitinase OTU domain-containing ubiquitin aldehyde-binding protein 1 (OTUB1) [96]. A subsequent study from our group demonstrated that OTUB1 is a *bona fide* FIH target protein, which is hydroxylated on asparagine 22 [31]. This hydroxylation impacts on OTUB1 substrate targeting, affecting in turn cellular energy metabolism [31]. This demonstrates functional relevance of the asparagine hydroxylation of this DUB and links OTUB1 with the regulation of energy metabolism. Another link between the hydroxylases and the ubiquitin pathway was made by the investigation of the interactome of PHD3 and FIH, demonstrating that especially FIH interacted with several proteins of the

ubiquitin system, including deubiquitinating enzymes [97]. It would be interesting to investigate these interactions in more detail.

An indirect effect of prolyl hydroxylation on a deubiquitinating enzyme was shown for USP9x, which no longer interacts with its target protein FOXO3a following FOXO3a hydroxylation by PHD1, affecting FOXO3a-dependent regulation of Cyclin D1 [30].

To our knowledge, a DUB regulating HIF hydroxylases has not been reported yet. But based on the identified interactions and the reported regulation of hydroxylases by E3 ligases, it seems likely that such regulations also occur.

3.2 HIF transcription factor subunits and deubiquitinases

HIFα subunits have been shown to be regulated by numerous different E3 Ub ligases. As DUBs oppose the function of E3 ligases, it is not too surprising that also several different DUBs have now been associated with the regulation of HIFα ubiquitination [25]. So far, DUBs from four out of the seven described DUB subfamilies have been identified to be involved in the regulation of HIFα (Fig. 2B). To our knowledge, it is currently unclear whether DUBs are affected by HIF subunits through protein:protein interaction.

Most of the deubiquitinases that regulate HIFα belong to the USP subfamily, with more than 50 enzymes the largest subfamily of DUBs [98]. HAUSP (USP7), USP8, USP28, VDU2 (USP20), USP9x, USP19 and USP52 have all been described to regulate HIF-1α. HAUSP has recently been reported as hypoxia-regulated DUB that stabilizes HIF-1α protein through deubiquitination [99]. Hypoxia induces K63-linked polyubiquitination of HAUSP, which enhances its activity towards HIF-1α [99]. Another member of the USP family, USP8, also deubiquitinates HIF-1α [100]. USP8 binds to the PAS domains of HIF-1α and HIF-2α and is implicated in the regulation of both proteins [100]. The USP8-mediated deubiquitination of HIF-1α has been linked to the regulation of ciliogenesis [100]. USP28 is a negative regulator of the GSK-3-dependent Fbw7-mediated polyubiquitination and subsequent degradation of HIF-1α, affecting cell migration and angiogenesis [66]. VDU2 (pVHL-interacting deubiquitinating enzyme 2) interacts with and deubiquitinates HIF-1α and

is itself ubiquitinated in a VHL-E3 ligase-dependent (but oxygen-independent) manner [101, 102]. USP9x affects the ubiquitination status of HIF-1α indirectly by regulating the degradation of VHL [103]. USP9x deubiquitinates Smurf1, an E3 ligase targeting VHL, protecting Smurf1 from degradation and leading to reduced VHL protein levels [103]. USP9x further affects glycolysis and cell proliferation through the regulation of VHL [103].

While the DUBs HAUSP, USP8, USP28, VDU2 and USP9x have been described to regulate HIFα by catalyzing the removal of Ub chains, the following DUBs of the USP protein family regulate HIF-1α through mechanisms independent of their enzymatic activity. USP19 interacts with the bHLH and PAS domain at the N-terminus of HIF-1α and reduction of USP19 expression impairs HIF-1α stabilization under hypoxic conditions [104]. The underlying mechanism remains to be uncovered [104]. A different mechanism of DUB-dependent regulation of HIF-1α has been reported for USP52, a non-protease homologue of USP that lacks a cysteine residue in its active site [105]. USP52 prevents HIF-1α mRNA degradation via its AU-rich 3'-UTR and co-localization with P-bodies (processing bodies), contributing to the mRNA stabilization and thereby indirectly to increased protein levels [106].

A member of the UCH family, UCHL1, interferes with the VHL-dependent polyubiquitination of HIF-1α through its interaction with and the deubiquitination of HIF-1α, promoting pulmonary metastases [107].

So far, the only DUB of the OTU family known to regulate HIF is OTUD7B, also called Cezanne. This DUB is specific for K11-linked Ub chains and negatively regulates K11-linked Ub chains of HIF-1α, affecting its degradation in a VHL-dependent but proteasome-independent manner [108]. Of note, these investigations identified a third type of Ub chains (K11-linked chains) that are attached to HIF-1α, but the responsible E3 ligase remains to be elucidated. Cezanne has further been linked to the regulation of the stability of the transcription factor E2F1, affecting *HIF2A* transcription independent of hypoxia [109]. Interestingly, it has been reported that Cezanne expression is induced in hypoxia but this might be cell type specific [108, 110].

The monocyte chemotactic protein-induced protein 1 (MCPIP1; also called Regnase-1) of the MCPIP family, a CCCH Zn finger–containing protein, deubiquitinates HIF-1α, preventing its degradation and impacting on HIF-1α target gene expression [111]. HIF-2α is affected by MCPIP1 on the transcript level and HIF-2α in turn also regulates MCPIP1 expression [112]. The underlying mechanisms are unclear, but MCPIP1 might regulate HIF-2α transcripts via its endonuclease activity [112].

4. Conclusions

Hypoxia represents a major threat to cellular energy homeostasis and survival. To enable cells to react appropriately to changes in oxygen supply, cellular oxygen sensing evolved, allowing for the adaptation of cellular energy metabolism and other biological processes to survive this environmental stress condition. The HIF pathway is the master regulator of the cellular adaptive response to low oxygen conditions and it is regulated on multiple levels by the ubiquitin system. Taking into account that the HIF pathway is a particularly rapid turnover system, it is likely more prone to changes in Ub-mediated stability than other pathways. Furthermore, the Ub-dependent regulation of hydroxylase protein levels provides an additional intervention point for the fine tuning of HIF-dependent gene expression due to the direct dependence of HIFα degradation and HIF-mediated transactivation on hydroxylase activity. But interestingly, PHD2 is the only hydroxylase that does not seem to be regulated by the ubiquitin system. This apparent uncoupling of the regulation of the HIF pathway from the ubiquitin system at this key HIF signaling node would be of interest for further investigations. The extent of the ubiquitin system-dependent regulations of the HIF pathway beside the well-characterized VHL-dependent mechanism is only beginning to be unraveled and it will be important to analyze the relative contributions of each of the here described mechanisms. Another key question concerns the relevance of the described HIF regulators under physiological conditions compared with pathophysiological conditions. Of note, it is emerging that the oxygen-sensing hydroxylases also affect proteins of the ubiquitin system, demonstrating a mutual regulation of these systems. This is especially important, as proteins

of the ubiquitin system often impact on a plethora of different biological processes and signaling pathways. Oxygen-dependent regulation of such promiscuous proteins seems to be especially suited, because a single hydroxylation event of such a protein could influence very quickly a large number of different processes, which can be of great advantage for the response to life-threatening environmental stress conditions such as hypoxia.

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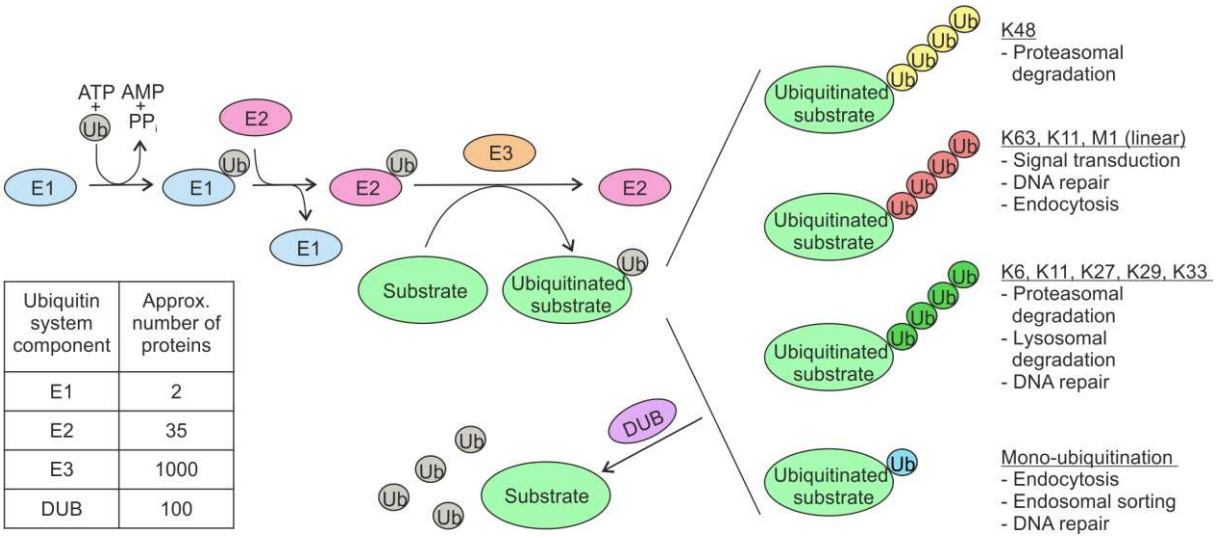
This review is dedicated to the memory of Lorenz Poellinger, a driving force in the field and a good friend.

Figures:



Ubiquitin system component	Approx. number of proteins
E1	2
E2	35
E3	1000
DUB	100

Fig. 1. The ubiquitin system. For the ubiquitination of a protein ubiquitin (Ub) is activated by an E1 Ub-activating enzyme, transferred to an E2 Ub-conjugating enzyme and covalently attached to a substrate protein by an E3 Ub ligase. This leads to the conjugation of a single Ub molecule (mono-ubiquitination) or ubiquitin chains (poly-ubiquitination) with isopeptide bonds between internal Ub lysine residues. Linear chains are generated through a linkage between the C-terminal glycine and the N-terminal methionine (M1) of Ub molecules. As indicated, differentially conjugated chains have various functional implications [22]. The opposing enzymes of E3 ligases are deubiquitinases (DUBs) that proteolytically remove Ub molecules or chains from substrate proteins. Approximate numbers of the components of the ubiquitin system are indicated in the table. PP_i, inorganic diphosphate.

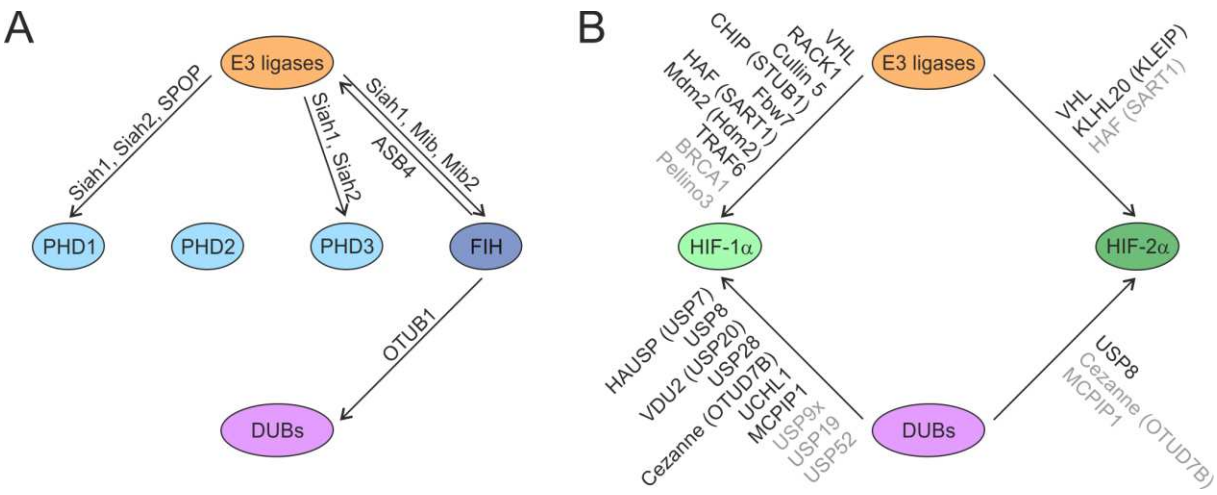


Fig. 2. Functional interplay between the HIF pathway and the ubiquitin system. The names of involved E3 ligases, proteins of E3 ligase complexes or DUBs are indicated along the arrows. **(A)** Mutual regulation of oxygen-sensing hydroxylases, E3 ligases and deubiquitinases (DUBs). Only direct regulations on the protein level were considered. **(B)** Regulation of HIFα subunits by E3 ligases and DUBs. Direct regulations of target proteins are indicated by black writing alongside the arrows, non-enzymatic and/or indirect regulations (e.g. of mRNA expression or stability) are indicated by grey writing. A HIFα-dependent regulation of E3 ligases or DUBs through protein:protein interaction has not been reported so far. HIF-dependent regulations of gene expression were not taken into account.

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